

The Endothelial Nitric Oxide Synthase Gene Variants as a Risk Factor for Chronic Lymphocytic Leukemia

Mustafa PEHLIVAN¹, Ayse G. TOMATIR², Ayşe F. NURSAL³, Handan H. SAHİN¹, Sacide PEHLIVAN⁴

¹ Gaziantep University Faculty of Medicine, Department of Haematology, Gaziantep

² Pamukkale University Faculty of Medicine, Department of Medical Biology, Denizli

³ Hitit University Faculty of Medicine, Department of Medical Genetics, Corum

⁴ Istanbul University Faculty of Medicine, Department of Medical Biology, Istanbul, TURKEY

ABSTRACT

Nitric oxide (NO) plays complicated roles in carcinogenesis. Endothelial nitric oxide synthase (eNOS) gene is responsible for most of the NO produced. For this reason, it was considered that the eNOS gene variants is associated with cancer susceptibility. The aim of this study was to determine whether eNOS variants (G894T and intron 4 VNTR a/b) affect in Chronic Lymphocytic Leukemia (CLL) risk in Turkish patients. This is a prospective single-center crosssectional study in patients with CLL. A total of 60 CLL patients and 100 healthy controls with similar age and sex were included to this study. Two eNOS gene variants (G894T and intron 4 VNTR a/b) were analysed with polymerase chain reaction (PCR) and/or restriction fragment length polymorphism (RFLP) methods. In this study, we found that the TT genotype of eNOS G894T variant was significantly associated with an increased risk in patient with CLL compared with control (OR: 0.867, CI: 0.785-0.957, $p=0.001$). There was not any significant difference in the eNOS G894T allele distribution between the groups ($p>0.05$). In addition, no significant difference was detected between the CLL patients and healthy controls with respect to the frequencies of genotypes and alleles in intron 4 VNTR a/b variant of eNOS. eNOS gene variants (G894T and intron 4 VNTR a/b) in CLL patients were simultaneously analyzed for the first time in present study. Our study suggest that the eNOS G894T variant may be associated with the development of CLL in the Turkish population.

Keywords: Chronic lymphocytic leukemia, Endothelial nitric oxide synthase, G894T, Intron 4 VNTR a/b

ÖZET

Kronik Lenfositik Lösemide Risk Faktörü Olarak Endotelial Nitrik Oksit Sentaz Gen Varyantları

Nitrik oksit (NO) karsinogeneizde kanşık role sahiptir. Endotelial nitrik oksit sentaz (eNOS) geni üretilen NO'un çoğundan sorumludur. Bu nedenle, eNOS gen varyantlarının kansere yatkınlık ile ilişkili olduğu varsayılmaktadır. Bu çalışmanın amacı kronik lenfositik lösemili (KLL) Türk hastalarında eNOS (G894T ve intron 4 VNTR a/b) varyantlarının risk faktörü olup olmadığı saptamaktır. Bu çalışma, KLL hastalarında prospektif, tek merkezli kesitsel bir çalışmadır. Bu çalışmanın amacı eNOS varyantlarının (G894T ve 4VNTR b/a) Türk hastalarda Kronik lenfositik lösemi (KLL) riskini etkileyip etkilemediğini saptamaktır. Bu çalışmaya 60 KLL hastası ve benzer yaş ve cinsiyette 100 sağlıklı kontrol dahil edilmiştir. İki eNOS gen varyantı (G894T ve intron 4VNTR a/b) polimeraz zincir reaksiyonu (PZR) ve/veya restriksiyon fragman uzunluk polimorfizmi (RFLP) yöntemleri ile analiz edildi. Bu çalışmada, eNOS G894T varyantı TT genotipinin KLL hastalarında kontrole göre belirgin şekilde artmış risk ile ilişkili olduğunu bulduk (OR: 0.867, CI: 0.785-0.957, $p=0.001$). eNOS G894T allel dağılımında gruplar arasında belirgin fark yoktu ($p>0.05$). Ayrıca eNOS intron 4' deki VNTR a/b varyant genotip ve allel sıklığı açısından KLL hastaları ve kontroller arasında belirgin fark saptanmadı. Bu çalışmada KLL hastalarındaki eNOS gen varyantları (G894T ve intron 4VNTR a/b) ilk defa eş zamanlı olarak incelenmiştir. Sonuçlarımız eNOS G894T varyantının Türk toplumunda KLL gelişmesi ile ilişkili olabileceğini önermektedir.

Anahtar Kelimeler: Kronik lenfositik lösemi, Endotelial nitrik oksit sentaz, G894T, Intron 4 VNTR a/b

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia type in the Western Europe and North America and characterized by proliferation and accumulation of morphologically mature CD5+/CD19+/CD23+ lymphocytes in the peripheral blood, bone marrow, and lymphoid tissues.^{1,2} The precise pathogenesis of CLL is still unknown. However genetic, environmental and immunological factors have been offered to account for the development of CLL. Patients with CLL represent a highly variable clinical course, with overall survival ranging from months to decades, which reflects the biological diversity of the disease.³ It was thought that there has been different signaling pathways associated with the onset and course of CLL.

Nitric oxide (NO), a free radical molecule, plays a crucial role in regulating cell death, angiogenesis, killing of tumor cells and reducing cell adhesion to endothelium.⁴ It is considered that the over production of NO is related to carcinogenesis. Nitric oxide synthase (NOS) is the major source of the NO production; conversion of L-arginine to L-citrulline take place during NO production.⁵ There are three different isoforms of NOS family. They include neuronal-NOS (nNOS/NOS1), inducible-NOS (iNOS/NOS2), and endothelial-NOS (eNOS/NOS3).⁶ The eNOS gene is located on chromosome 7 (7q36) and contains 26 exons, spanning 21 kilobases.⁴ The eNOS plays a role in regulation of platelet aggregation, leukocyte adherence, blood pressure, and vascular smooth muscle cell mitogenesis and angiogenesis. The eNOS have various polymorphic sites. One of three functional variants, the G894T variant (rs1799983) is in exon 7 and results in a Glu-Asp substitution during the protein synthesis. Other variant of eNOS is a functional variable number tandem repeats (VNTR) (27 bp repeat, intron 4 VNTR a/b) which causes basal NO production.⁷ Although there was several studies evaluated the impact of eNOS variants on human cancer risk, the results are still remain unclear. In this context, we evaluated the possible role of the G894T and intron 4 VNTR a/b variants of eNOS gene on development of CLL in Turkish patients.

PATIENTS AND METHODS

Subjects

Sixty CLL patients (35 males and 25 females) were diagnosed at Gaziantep University, Medical Faculty, Department of Hematology and 100 healthy controls (51 males and 49 females) were recruited in this study. Clinical characteristics, peripheral blood morphologies, immuno-phenotype, and B-lymphocytes count of higher than $5.0 \times 10^9/L$, confirmed the diagnosis of CLL.⁸ Healthy controls were recruited from the same geographical areas with patients, and they were well-matched with the patients group in terms of gender, age and ethnicity. The control group was selected from the volunteers who did not have any chronic disease. All participants were given detailed verbal and written information regarding the purpose and procedures of the study. This study was conducted under guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by Local Ethics Committee (07-2007/40).

Genotyping Analysis

About 5 mL peripheral blood was collected by venipuncture using Vacutainer tubes with EDTA as anticoagulant for the analysis of eNOS gene variant. DNA extracted from leukocytes according to the established protocol.⁹ Two eNOS gene variants (G894T and intron 4 VNTR a/b) were determined by polymerase chain reaction (PCR) and/or restriction fragment length polymorphism (RFLP) as described by Safarinejad et al.¹⁰ The G894T variant was determined by PCR using the following primers 5'-CATGAGGCTCAGCCCCAGAAC-3' (forward) and 5'-AGTCAATCCCTTTGGTGCTCAC-3' (reverse). PCR products were digested by MboI enzyme (Invitrogen, Carlsbad, CA, USA) at 37°C for overnight. Then, fragments were separated on 3% agarose gel electrophoresis and visualized using ultraviolet light. The 206 bp products had a consistent restriction site resulting in a 119 bp and an 87bp fragment. The 27 bp-VNTR a/b genotype in intron 4 of eNOS were evaluated by PCR amplification using primers 5'-AGGCCCTATGTAGTGCCTTT-3' (forward) and 5'-TCTCTTAGTGCTGTGGTCAC-3' (reverse).¹⁰ The amplified products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium

Table 1. Genotype and allele distribution of eNOS gene variants in CLL patients and control subjects.

		CLL n ^a (%)	Control n ^b (%)	OR	95%CI	p
eNOS (4a/b)	Genotype					
	AA	38 (63.3)	72 (72)	0.672	0.339-1.330	0.292
	AB	16 (26.7)	25 (25)	0.917	0.442-1.901	0.853
	BB	6 (10.0)	3 (3.0)	0.278	0.067-1.158	0.081
	Allele					
	A	92 (76.7)	129 (64.5)			
	B	28 (23.3)	31 (35.5)	0.596	0.337-1.055	0.100
eNOS(G894T)	Genotype					
	GG	34 (56.7)	65 (65.0)	0.808	0.417-1.563	0.612
	GT	18 (30.0)	35 (35.0)	1.256	0.631-2.501	0.604
	TT	8 (13.3)	0 (-)	0.867	0.785-0.957	0.001
	Allele					
	G	86 (71.6)	165 (82.5)			
	T	34 (28.3)	45 (17.5)	0.726	0.432-1.218	0.230

OR: Odds Ratio; CI: Confidence Intervals; ^an: 60, ^bn: 100, Adjusted by age and gender

bromide staining. The wild type allele contained five tandem repeats of 27 bp and 420 bp and the mutant allele four tandem repeats of 27 bp and 393 bp band.

Statistical Analysis

All data were analyzed using software SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL; USA). The statistical significance of the differences between the patient and control groups was estimated by logistic regression analysis. Odds ratio (OR) and 95% confidence interval (CI) were also calculated. Differences in eNOS genotype frequencies between the patient and control groups were compared with chi-square test and, Fisher's exact test was used when needed. All analyses were two-tailed, and differences were interpreted as statistically significant when $p < 0.05$.

RESULTS

In the present study, a total of 160 subjects, including 60 CLL patients and 100 adult healthy controls were genotyped for the G894T and intron 4 VNTR4 a/b variants of eNOS gene. There were 25 (41.6%) women and 35 (58.4%) men in the patient group and 49 (49%) women and 51 (51%) men in the control group. The genotype distributions and allele frequencies of eNOS G894T and intron 4VNTR a/b variants among the patients and controls are shown in Table 1.

The prevalence of genotypes of AA, AB, and BB profiles for eNOS intron 4 VNTR variant were 63.3%, 26.7% and 10% respectively in patients with CLL, and 72%, 25% and 3% respectively in control group. No significant differences were observed between groups for the eNOS intron 4 VNTR genotype and allele frequencies.

For the G894T variant of the eNOS gene, in the CLL group 34 patients (56.7%) were GG genotype, 18 patients (30%) were GT genotype, and 8 patients (13.3%) were TT genotype; in the control group, 65 healthy individuals (65%) were GG genotype, 35 individuals (35%) were GT genotype, and 0 individuals (0%) were TT genotype. The genotype distribution of eNOS G894T variant was showed statistically significant difference between the patients and the controls. We found that the TT genotype of eNOS G894T variant was significantly associated with an increased risk in patient with CLL compared with healthy control ($p = 0.001$, OR:0.867, CI: 0.785-0.957). There was not any significant difference in the eNOS G894T allele distribution between the patients and controls ($p > 0.05$).

DISCUSSION

CLL is a rather heterogenous malignant disease in terms of clinical presentation, and response to treatment, and remains untreatable with conven-

tional chemotherapy.¹¹ The data indicate that a strong family and genetic factors contribute to the etiology of CLL.¹²

NO, an important messenger molecule, is involved several physiologic and patho-physiologic processes such as neurotransmission, smooth muscle relaxation, immunity, vasodilatation, and carcinogenesis.¹³ In previous studies, it was reported that over production of NO can cause DNA damage and inhibits DNA repair mechanism.^{14,15} However, in some other studies it was reported that NO can increase the activity of DNAPKCs (the catalytic subunit of the DNA-dependent protein kinase complex), thus preventing DNA damage; furthermore, it may enhance blood flow, destroy tumor cells, diminish tumor cell adhesion to endothelium and regulate apoptosis.¹⁶⁻¹⁹ In animal experiments, also it was shown that NO has anti-tumor effects produced by the immune-defense system.²⁰

Since NO production can be affected by eNOS gene, eNOS gene variants have been studied in carcinogenesis. It has been shown that eNOS gene can modulate cancer-related events such as angiogenesis, invasion, and metastasis.²¹ Recent studies suggest that the expression of eNOS is not restricted to normal tissues, and it is also widely expressed in tumor tissues, adjusting endothelial cell damage and mobilization and homing of bone marrow endothelial progenitor cells, and participating in the formation of blood vessels through NO generation, which can have an impact on the growth and metastasis of tumors.²²

Angiogenesis is involved in solid tumor growth, progression and metastasis. Evidence supports that the progression of hematolymphoid malignancies depend on the formation of new blood vessel. Vascular endothelial factor (VEGF) is the main proangiogenic agent that stimulates receptors on vascular endothelial cells and induces new blood vessel formation.²³ eNOS has a central mediator role in VEGF-induced angiogenesis and vascular permeability.²⁴ Demacq et al. reported that VEGF and eNOS gene variants are associated with high risk of relapse in childhood with acute lymphoblastic leukemia (ALL).²⁵ Almost 400 eNOS variants have been described up to now and it was found that some of them to be related to carcinogenesis.⁵ It was reported that some of these variants are sig-

nificantly related with the development of certain cancer types.^{26,27}

This case-control study examined the G894T and intron 4 a/b variants of eNOS gene and their relationship to susceptibility for CLL in a Turkish cohort. Although association of the eNOS variants with several solid tumors has been previously shown, as yet, there is no study in literature about the association of this eNOS variants with hematologic diseases. To our knowledge, this is the first study to evaluate the eNOS variants in relation to CLL risk in a Turkish cohort.

VNTR variant in intron 4 of eNOS gene accounts for >25% of basal plasma NO generation.¹⁰ There have been existed two alleles in intron 4 of the eNOS gene. While the larger allele consists of five tandem repeat 27-bp repeats, small allele has four repeats.²⁸ It was reported that carriers of the 4a allele had lower NO levels than 4b/4b homozygous subjects.²⁹ The impact of 4 a/b variant of the eNOS gene is still unclear, it has been declared that this variant would regulate the expression of the eNOS gene by the editing of small RNAs (siRNAs).³⁰ Phenotypic significance of the eNOS intron 4 a/b variant was evaluated in various conditions in previous studies.³¹⁻³³ It was found that 4a allele is associated with coronary artery disease, stroke, and renal disease.³⁴⁻³⁶ In studies for cancer susceptibility, it was found that eNOS 4 a/b variant is associated with colorectal cancer, prostate cancer, whereas there was no significant association with bladder cancer and overall cancers.^{4,7,10,37,38} In sub-group analysis based on ethnicity of meta analysis, it was reported that the eNOS 4 a/b variant was associated with an increased risk of cancer in Caucasians.²⁴ In our study, we found that the eNOS intron 4 a/b variant wasn't significantly different between patients and controls.

Effects of the eNOS G894T variant on cancer risk and progression have been investigated by several researchers and findings of these studies have been heterogeneous and sometimes contradictory. Although, there were studies that the eNOS G894T variant was associated with prostate cancer, bladder cancer, colorectal cancer, larynx cancer, there were studies that the eNOS G894T variant was not associated with cancer and survival.^{4,5,7,39-42} Lee et al. declared that there was no significant associa-

tion between the eNOS G894T variant and breast cancer, but this variant was associated with lymph node involvement.⁴³

In present study, we found a significant difference in genotype distribution between patients and controls. We found that TT genotype of eNOS G894T was significantly higher in patients with CLL compared with controls ($p=0.001$).

Case-control association studies represent an important tool for understanding the role that genes play in the carcinogenesis. The limitations of this study should also be noted. First, we focused on only two variants involved in the pathway of eNOS, other regulatory genes in the NOS family signaling pathway may also contribute to the pathogenesis of CLL. Second, owing to the relatively small sample size, the frequencies of some homozygous variants were low in groups and therefore reduced the statistical power. Finally, lack of assessment of expression levels of eNOS is also a limitation of this study.

CONCLUSIONS

Although findings with respect to the role of eNOS gene variants in cancer pathogenesis are conflicting, the difference between data may be explained in view of the genetic variability of populations. This study did not reveal significant association between eNOS intron 4 VNTR variant and CLL, however we represented the first result on eNOS G894T variant and CLL in a Turkish cohort. Large-scale studies should be replicated with different subjects and/or other ethnic groups to fully elucidate the effects of these variants on susceptibility to CLL.

Acknowledgements:

We should like to thank all of the participants for their support. We thank the DNA data bank (funded by Gaziantep University Research Foundation Grant reference TF.07.10) for the DNA samples.

REFERENCES

- Hus I, Rolinski J. Current concepts in diagnosis and treatment of chronic lymphocytic leukemia. *Contemp Oncol (Pozn)* 19: 361-367, 2015.
- Landau DA, Wu CJ. Chronic lymphocytic leukemia: molecular heterogeneity revealed by high-throughput genomics. *Genome Med* 5: 47, 2013.
- Vural F, Karaca E, Soyer N, et al. Comparison of CD38, ZAP70 and hTERT Expression with Known Prognostic Markers in Patients with Chronic Lymphocytic Leukemia During Five-Year Follow-up Period. *UHOD* 3: 179-184, 2014.
- Haque S, Mandal RK, Akhter N, et al. G894T and 4a/b polymorphisms of NOS3 gene are not associated with cancer risk: a meta-analysis. *Asian Pac J Cancer Prev* 16: 2929-2937, 2015.
- Yanar K, Çakatay U, Aydın S, et al. Relation between Endothelial Nitric Oxide Synthase Genotypes and Oxidative Stress Markers in Larynx Cancer. *Oxid Med Cell Longev* 2016: 4985063, 2016.
- Albrecht EW, Stegeman CA, Heeringa P, et al. Protective role of endothelial nitric oxide synthase. *Journal of Pathology* 199: 8-17, 2003.
- Polat F, Diler SB, Azazi I, Öden A. T-786C, G894T, and intron 4 VNTR (4a/b) polymorphisms of the endothelial nitric oxide synthase gene in bladder cancer cases. *Asian Pac J Cancer Prev* 16: 2199-2202, 2015.
- Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* 111: 5446-5456, 2008.
- Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16: 1215, 1988.
- Safarinejad MR, Safarinejad S, Shafiei N, Safarinejad S. Effects of the T-786C, G894T, and intron 4 VNTR (4a/b) polymorphisms of the endothelial nitric oxide synthase gene on the risk of prostate cancer. *Urol Oncol* 31: 1132-1140, 2013.
- Routledge DJ, Bloor AJ. Recent advances in therapy of chronic lymphocytic leukaemia. *Br J Haematol* 174:351-367, 2016.
- Rossi D, Gaidano G. The clinical implications of gene mutations in chronic lymphocytic leukaemia. *Br J Cancer* 114: 849-854, 2016.
- Lu J, Wei Q, Bondy ML, et al. Promoter polymorphism (-786t>c) in the endothelial nitric oxide synthase gene is associated with risk of sporadic breast cancer in non-Hispanic white women age younger than 55 years. *Cancer* 107: 2245-2253, 2006.
- Chien YH, Bau DT, Jan KY. Nitric oxide inhibits DNA-adduct excision in nucleotide excision repair. *Free Radic Biol Med* 36: 1011-1017, 2004.
- Jadeski LC, Chakraborty C, Lala PK. Nitric oxide-mediated promotion of mammary tumour cell migration requires sequential activation of nitric oxide synthase, guanylate cyclase and mitogen-activated protein kinase. *Int J Cancer* 106: 496-504, 2003.
- Xu W, Liu L, Smith GC, Charles IG. Nitric oxide upregulates expression of DNA-PKcs to protect cells from DNA-damaging anti-tumour agents. *Nat Cell Biol* 2: 339-345, 2000.
- Dhar A, Brindley JM, Stark C, et al. Nitric oxide does not mediate but inhibits transformation and tumor phenotype. *Mol Cancer Ther* 2: 1285-1293, 2003.

18. Kong L, Dunn GD, Keefer LK, Korthuis RJ. Nitric oxide reduces tumor cell adhesion to isolated rat postcapillary venules. *Clin Exp Metastasis* 14: 335-343, 1996.
19. Choi BM, Pae HO, Jang SI, et al. Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. *J Biochem Mol Biol* 35: 116-126, 2002.
20. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 15: 323-350, 1997.
21. Jadeski LC, Hum KO, Chakraborty C, Lala PK. Nitric oxide promotes murine mammary tumour growth and metastasis by stimulating tumour cell migration, invasiveness and angiogenesis. *Int J Cancer* 86: 30-39, 2000.
22. Han JG, Gao LB, Zhang J, et al. Relationship between Polymorphisms of Angiotensin-converting Enzyme and Endothelial Nitric Oxide Synthase Gene Intron 4 VNTR and Risk for Cervical Cancer. *Life Science Journal* 9: 100-104, 2012.
23. Song G, Li Y, Jiang G. Role of VEGF/VEGFR in the pathogenesis of leukemias and as treatment targets (Review). *Oncol Rep* 28: 1935-1944, 2012.
24. Gao X, Wang J, Wang W, et al. eNOS Genetic Polymorphisms and Cancer Risk: A Meta-Analysis and a Case-Control Study of Breast Cancer. *Medicine (Baltimore)* 94(26): e972, 2015.
25. Demacı C, Vasconcellos VB, Izidoro-Toledo TC, et al. Vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (NOS3) polymorphisms are associated with high relapse risk in childhood acute lymphoblastic leukemia (ALL). *Clin Chim Acta* 411: 1335-1340, 2010.
26. Terrazzino S, La Mattina P, Masini L, et al. Common variants of eNOS and XRCC1 genes may predict acute skin toxicity in breast cancer patients receiving radiotherapy after breast conserving surgery. *Radiotherapy and Oncology* 103: 199-205, 2012.
27. Heffer LA, Ludwig E, Lampe D, et al. Polymorphisms of the endothelial nitric oxide synthase gene in ovarian cancer. *Gynecologic Oncology* 86: 134-137, 2002.
28. Buraczynska M, Ksiazek P, Zaluska W, et al. Endothelial nitric oxide synthase gene intron 4 polymorphism in patients with end-stage renal disease. *Nephrol Dial Transplant* 19: 2302-2306, 2004.
29. Sener EF, Emirogullari ON, Serhattioglu F, Ozkul Y. The role of endothelial nitric oxide synthase gene G894T and intron 4 VNTR polymorphisms in hemodialysis patients with vascular access thrombosis. *Anadolu Kardiyol Derg* 14: 239-243, 2014.
30. Ramírez-Patiño R, Figueroa LE, Puebla-Pérez AM, et al. Intron 4 VNTR (4a/b) polymorphism of the endothelial nitric oxide synthase gene is associated with breast cancer in Mexican women. *J Korean Med Sci* 28: 1587-1594, 2013.
31. Sivri N, Unlu A, Palabişik O, et al. Endothelial nitric oxide synthase intron 4a/b polymorphism in coronary artery disease in Thrace region of Turkey. *Biotechnol Biotechnol Equip* 28: 1115-1120, 2014.
32. Nasr HB, Dimassi S, M'hadhbi R, et al. Functional G894T (rs1799983) polymorphism and intron-4 VNTR variant of nitric oxide synthase (NOS3) gene are susceptibility biomarkers of obesity among Tunisians. *Obes Res Clin Pract* 10: 465-475, 2016.
33. Celik U, Yildizdas D, Alhan E, et al. Genetic dilemma: eNOS gene intron 4a/b VNTR polymorphism in sepsis and its clinical features in Turkish children. *Turk J Pediatr* 50: 114-119, 2008.
34. Wang XL, Sim AS, Badenhop RF, et al. A smoking dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. *Nat Med* 2: 41-45, 1996.
35. Hou L, Osei-Hyiaman D, Yu H, et al. Association of a 27 bp repeat polymorphism in eNOS gene with ischemic stroke in Chinese Population. *Neurology* 56: 490-496, 2001.
36. Wang Y, Kikuchi K, Suzuki H, et al. Endothelial Nitric Oxide Synthase gene Polymorphism in intron 4 affects the progression of renal failure in Non - diabetic renal diseases. *Nephrol Dial Transplant* 14: 2898-2902, 1999.
37. Yeh CC, Santella RM, Hsieh LL, et al. An intron 4 VNTR polymorphism of the endothelial nitric oxide synthase gene is associated with early-onset colorectal cancer. *Int J Cancer* 124: 1565-1571, 2009.
38. Wu X, Wang ZF, Xu Y, et al. Association between three eNOS polymorphisms and cancer risk: a meta-analysis. *Asian Pac J Cancer Prev* 15: 5317-5324, 2014.
39. Zhang Y, Jia Q, He Q, et al. The Glu298Asp polymorphism in the NOS3 gene and the risk of prostate cancer. *Tumour Biol* 35: 4735-4739, 2014.
40. Verim L, Toptas B, Ozkan NE, et al. Possible relation between the NOS3 gene GLU298ASP polymorphism and bladder cancer in Turkey. *Asian Pac J Cancer Prev* 14: 665-668, 2013.
41. Arkan S, Cacina C, Guler E, et al. The effects of NOS3 Glu-298Asp variant on colorectal cancer risk and progression in Turkish population. *Mol Biol Rep* 39: 3245-3249, 2012.
42. Fu H, Zhang Y, Jin L, Ding Z. Endothelial nitric oxide synthase (eNOS) G894T polymorphism is not associated with breast cancer risk: new evidence. *Breast Cancer Res Treat* 128: 595-598, 2011.
43. Lee KM, Choi JY, Lee JE, et al. Genetic polymorphisms of NOS3 are associated with the risk of invasive breast cancer with lymph node involvement. *Breast Cancer Res Treat* 106: 433-438, 2007.

Correspondence:

Dr. Ayşe Feyda NURSAL
 Hitit Üniversitesi, Tıp Fakültesi
 Tıbbi Genetik Anabilim Dalı
 Fen-Edebiyat Fakültesi Yerleşkesi
 Ulukavak Mahallesi
 CORUM / TURKEY

 Tel: (+90-364) 222 11 00
 Fax: (+90-364) 222 11 02
 e-mail: feydanursal@hotmail.com